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Hepatitis C Virus: Quantitation and Distribution in Liver

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The optimal method for viral quantitation and the most appropriate site for determining viral load in patients with chronic hepatitis C virus (HCV) infection are unknown. We developed a method for measuring HCV RNA in the liver with the following features: 1) efficient extraction of RNA from tissue (89% of RNA recovered); 2) accurate amplification using branched DNA with strong concordance between a single sample tested on multiple occasions either in the same or in different runs; 3) good sensitivity (95%) and specificity (100%). HCV RNA was detected in as little as 2 mg of tissue, and viral load determined in a needle biopsy was representative of viral load in other parts of the liver. Within individual livers, 68% of the samples quantitated were within 1.5-fold of the geometric mean, and 95% were within 2.2-fold of the geometric mean. The mean ratio of virus in the liver and serum was 103, range 17.4–286. A delay of 30 minutes before freezing the liver tissue resulted in a reduction in the measured viral load in some, but not all instances. A sensitive, specific, and reproducible method for quantitating HCV RNA in the liver has been developed. Measurement of viral load at one site was representative of viral load at other sites. While hepatic HCV RNA levels are consistently greater than serum levels, the ratio of liver to serum viral load varies widely. The clinical use of measurement of viral load in the liver remains to be defined. ***J. Med. Virol. 51: 217–224, 1997.***

KEY WORDS: hepatitis C virus; liver; viral quantitation

INTRODUCTION

Quantification of hepatitis C virus (HCV) RNA in human liver may be useful for the study of the pathogenesis of HCV-associated liver disease, and in the selection of patients with HCV who may be suitable candidates for treatment of their disease. If HCV was directly cytopathic to liver cells, one would predict a correlation between the amount of virus present and the degree of liver damage. Studies which have examined the relationship between HCV viral load (determined from measurement in serum) and the severity of liver disease have yielded conflicting results [Gretch et al., 1994; Nousbaum et al., 1995; Magrin et al., 1994]. The lack of consistent results may be due to different methods used for quantitating viral load in different studies, to suboptimal sample handling which may result in degradation of virus, and to the quantitation of virus in serum rather than in liver tissue, the latter being the major site of viral production. Finally, in the absence of reference standards for quantitation of unknown virus, comparison of viral load between different studies is problematic.

The importance of viral load in the management of patients with chronic HCV infection has been highlighted by several studies which have shown that lower levels of viremia are associated with a better response to interferon treatment [De Medina and Schiff, 1995; Fried and Hoofnagle, 1995]. However, many of the limitations of methods of viral quantitation outlined above also apply to studies of treatment responses. Whether measurement of viral load in tissue is superior to that in serum when investigating issues of pathogenesis and predicting response to treatment is unknown. However, before such issues can be addressed, there

Abbreviations used: bDNA, branched DNA; CAH/C, chronic active hepatitis with cirrhosis; CPH, chronic persistent hepatitis; HBV, hepatitis B virus; HCV, hepatitis C virus; RT-PCR, reverse transcriptase followed by polymerase chain reaction; HBsAg, hepatitis B surface antigen.

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TABLE I. Clinical and Laboratory Data on Liver Transplant Patients

Patient no.	Age (years)	Gender	Total bilirubin (mg/dL)[a]	ALT (IU/L)[b]	Albumin (g/dL)[c]	PT (sec)[d]	INR[e]	Risk factor
1	54	M	47.7	172	2.7	15.3	1.8	Unknown
2	44	F	0.5	1963	3.9	14.3	1.6	Blood transfusion
3	42	M	3.41	58	2.9	12.6	1.2	Unknown
4	65	M	39.3	72	3.0	19.1	2.8	Unknown
5	61	M	2.1	64	3.0	14.2	1.6	Unknown

[a]Normal value for total bilirubin 0.1–1.2 mg/dL
[b]Normal ALT value 9–50 IU/L.
[c]Normal albumin value 3.4–4.7 g/dL.
[d]Normal prothrombin time (PT) 10.3–13.3 seconds.
[e]International Normalized Ratio (INR) 1.0.

remain questions relating to the validity of sampling liver tissue obtained by needle biopsy (i.e., whether a specimen obtained by needle biopsy is representative of viral load throughout the liver), the method used for processing liver specimens once they have been obtained (i.e., how rapidly specimens need to be frozen once they are obtained to prevent inaccuracies in measured virus), and the methods used for extraction and quantitation of tissue RNA (i.e., whether the amount of virus measured after nucleic acid extraction reflects accurately the amount of virus present initially). This study addresses these issues.

MATERIALS AND METHODS

Liver and Serum Specimens

Study population. Five patients with end-stage HCV infection undergoing orthotopic liver transplantation at the University of California, San Francisco, were studied prospectively (see Table I). HCV infection was defined as follows: presence in serum of anti-HCV (EIA2 positive, Abbott Laboratories, N. Chicago, IL) and HCV RNA (by qualitative reverse transcriptase followed by polymerase chain reaction [RT-PCR]) [Wright et al., 1992], and absence of hepatitis B surface antigen (HBsAg). Biochemical markers of liver function were obtained at the time of transplantation. All patients gave informed consent to participate in hepatitis research. In addition, the serum and explant tissue from 40 patients with end-stage liver disease who had undergone liver transplantation were used to develop methods for viral quantitation in the liver; 20 patients had HCV infection (positive controls), 5 patients had hepatitis B virus (HBV) infection, and 15 patients had nonviral liver disease (negative controls).

Viral quantitation and liver histology. For prospective evaluation of the distribution of viral RNA within the liver, needle biopsies were obtained from seven different sites in the explants from each of five patients. A Jamshedi needle was used to obtain biopsies from the caudate lobe, the superior, middle, and inferior parts of the left lobe, and the superior, middle, and inferior parts of the right lobe within 30 minutes of liver removal. Specimens for HCV RNA quantification were immediately frozen using liquid nitrogen and petroleum ether, and maintained on dry ice until storage at −80°C. In order to assess the effects of tissue preservation on viral quantitation, additional biopsies were obtained from the same seven sites in explanted livers from four of five patients, after a delay of 30 minutes. The weight of the biopsies ranged from 9 to 84 mg (mean 21 mg, median 18 mg). Serum specimens were obtained at the time of transplantation from two of the five patients in whom multiple liver specimens were available. Serum was separated from red blood cells within an hour of phlebotomy and frozen at −80°C to avoid prolonged exposure of the virus to the clot [Cuypers et al., 1992].

Parallel samples for histology were obtained simultaneously from the seven sites in the liver, preserved in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histopathological diagnosis was performed in a blinded fashion by a single pathologist (L.F.). The degree of portal, periportal, and lobular inflammation, and the degree of fibrosis was graded on a scale from 0 to 4 in accordance with recommended nomenclature [Ludwig, 1993; Scheuer, 1991]. All biopsy specimens were classified as showing chronic hepatitis with cirrhosis.

In the 40 patients used to develop the methods of viral quantitation in the liver, wedge biopsies were obtained within 5 minutes of removal of the liver, placed on dry ice within 1 hour, and stored at −80°C. Simultaneous serum samples were available in 16 of 20 HCV-infected patients and in all 20 non-HCV infected patients, but since these serum samples were assayed retrospectively, methods of specimen handling could not be validated.

Liver tissue processing. Liver specimens were processed using a modification of the procedure described by Cox [1968]. Frozen liver tissue, from both wedge and needle biopsies, was maintained on dry ice to prevent thawing during weighing prior to homogenization. Liver tissue was homogenized rapidly in cold solution (8 M guanidine-HCl [Sequenal Grade, Pierce Chemical Co., Rockford, IL], 0.2 M sodium acetate). The volume of homogenization solution was 0.5 mL for specimens weighing ≤25 mg, and 0.5 mL plus 20 μL for each 1 mg over 25 mg for specimens weighing >25 mg. Needle biopsy specimens were homogenized using a disposable Pellet Pestle Mixer and matching micro-

tubes (Kontes Inc., Owens, IL). Wedge biopsy specimens were homogenized using sterile disposable tissue grinders (Sage Products Inc., Crystal Lake, IL).

Homogenized liver tissue (0.5 mL) was transferred to large-volume microcentrifuge tubes and mixed with 25 μL 10% (w/v) N-lauroyl-sarcosine (Sarcosyl, Sigma Chemical Co., St. Louis, MO) and 10 μL 10 mg/mL poly A (poly [A], potassium salt, Sigma). To precipitate RNA, 250 μL 100% ethanol was added to the homogenized liver tissue mixture which then was vortexed vigorously and incubated overnight at −18 to −22°C. Precipitated RNA was collected by centrifugation in a microcentrifuge at 12,000*g* for 20 minutes at 2–8°C. The supernatant was removed and 500 μL of 70% ethanol was added to the pellet, which was then vortexed and centrifuged as described above. After aspiration of the supernatant, the remaining liquid was removed by rotary evaporation. In some experiments, solutions of 10 mg/mL poly U (Pharmacia Biotech, Piscataway, NJ), poly I (Sigma), poly I:C (Boehringer Mannheim Biochemicals, Indianapolis, IN), or poly A:U (U.S. Biochemical Co., Cleveland, OH) were prepared in distilled water and substituted for the 10 mg/mL poly A solution.

Detection of Viral RNA

HCV RNA quantification. HCV RNA was quantified in both serum and extracted liver tissue using the Quantiplex® HCV RNA branched DNA (bDNA) 1.0 assay (Chiron Corporation, Emeryville, CA) according to the manufacturer's instructions [Urdea, 1993; Sherman et al., 1993]. Results were expressed as HCV RNA milliequivalents per milliliter for serum specimens and HCV RNA milliequivalents per gram wet weight for liver tissue. One equivalent was defined as the amount of HCV RNA that generates a level of light emission equivalent to that generated by one copy of quality level 1 RNA standard [Collins et al., 1995]. The quantification limit of the Quantiplex® HCV RNA 1.0 assay for serum specimens is 0.35 mEq/mL [Kolberg et al., 1994]. Homogenates of wedge liver biopsies were divided into 500 μL aliquots, frozen, and stored at −80°C for use in multiple bDNA assay runs. In some experiments, liver biopsy specimens were tested with and without the addition of target probes to mediate capture of RNA. All bDNA assay runs included both positive and negative controls for serum or liver tissue. Control liver samples were processed in a manner identical to that of other samples.

HCV RNA detection by RT-PCR. HCV RNA was detected in serum specimens using a nested RT-PCR assay as described previously [Wright et al., 1992]. Comparison to a serially diluted sample quantitated in the HCV RNA 1.0 assay for genotype 1 indicated that this PCR assay was able to detect reliably 600 equivalents of viral RNA.

Nucleic acid standards. The procedure for processing liver tissue was optimized using ^{32}P-radiolabeled nucleic acids to monitor the recovery of HCV RNA and cellular DNA at each step. A ^{32}P-labeled RNA transcript containing the first 4,000 nucleotides of HCV-1 was prepared using the MEGAscript *in vitro* transcription kit for large scale synthesis of RNA (Ambion, Austin, TX), alpha-^{32}P-GTP (New England Nuclear, Beverly, MA), and a vector containing the entire HCV-1 sequence as described [Collins et al., 1995]. After ethanol precipitation, the purity of the RNA transcripts was estimated to be approximately 93% using DE81 chromatography [Sambrook et al., 1989]. ^{32}P-labeled DNA was prepared with heat-denatured, sonicated salmon sperm DNA (phenol extracted, Pharmacia, Piscataway, NJ) and alpha-^{32}P-dCTP (New England Nuclear) using the Oligo labeling kit (Pharmacia) according to the manufacturer's instructions.

Statistical Methods

Statistical analysis was performed using Statistica for Windows (Statsoft, Inc., Tulsa, OK) with the exception of the mixed-effects analysis of variance which was run using SAS-JMP (SAS Institute, Inc., Cory, NC). For statistical analysis, HCV RNA levels in both serum and liver samples were log transformed and samples below the detection limit were assigned a value of one half the serum detection limit (0.175 × mEq/mL). Results are expressed as the geometric mean with the range or the geometric mean with the standard deviation.

RESULTS

Optimization of RNA Extraction Method

Ethanol precipitation. Purification of HCV RNA was optimal when 0.5× volume of ethanol was included in the initial precipitation step for processing liver tissue. This resulted in recovery of 88.7% of ^{32}P-HCV RNA and removal of 93.9% of ^{32}P-DNA. Increasing volumes of ethanol did not improve the yield of ^{32}P-HCV RNA, but did increase the amount of contaminating ^{32}P-DNA (data not shown). Since 0.5× volume of ethanol yielded maximal recovery of HCV RNA with minimal contamination by cellular DNA, this method was used in subsequent experiments.

Efficiency of HCV RNA recovery. The efficiency of HCV RNA recovery from liver tissue was evaluated by adding known quantities of ^{32}P-radiolabeled synthetic transcripts or known quantities of viral RNA (derived from serum) to noninfected liver tissue. The efficiency was calculated by comparing the amount added initially with the amount recovered at the end, after processing liver specimens according to the standard protocol. When four liver homogenates were processed after addition of synthetic RNA, recovery of ^{32}P-HCV RNA was near complete (82.5%, 78.1%, 76.2%, and 85.9%, respectively, mean value of 80.7%). Five different dilutions of HCV positive serum were added to HCV negative liver homogenates, and the amount of recovered HCV RNA was compared with the amount of HCV RNA added. The mean recovery of HCV RNA from the liver homogenate was 79.0% (range 70.4–92.0%).

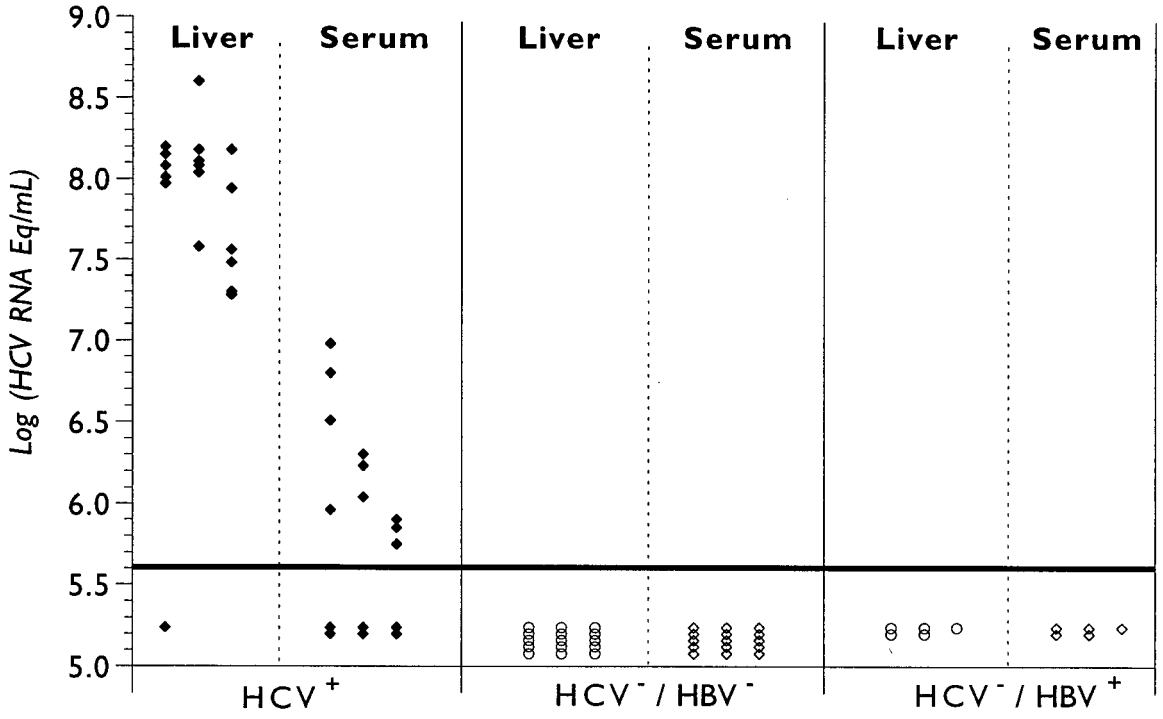

Fig. 1. Quantification of HCV RNA in serum and liver from patients with and without HCV infection. HCV RNA levels were measured with the bDNA assay in liver wedge biopsies and serum from liver transplant patients known to be HCV seropositive and HCV RNA positive by RT-PCR (HCV+), HCV seronegative/HBsAg negative (HCV–/HBV–), and HCV seronegative/HBsAg positive (HCV–/HBV+).

Quantification of Hepatic Viral RNA

Sensitivity and specificity of viral quantitation. HCV RNA was detectable by bDNA in 19 of 20 (95%) wedge biopsies and in 10 of 16 (62.5%) matching serum specimens from HCV seropositive patients, but was not detectable in either the serum or liver tissue from the 20 HCV seronegative patients (Fig. 1). As additional negative controls, liver specimens from three HCV seropositive and three HCV seronegative patients were processed by the standard protocol and tested by the bDNA assay without probes to mediate capture. No specimens yielded positive signals (data not shown).

In the HCV positive patients, mean liver HCV RNA levels were 87 mEq/g (range 19–400 mEq/g) and mean serum HCV RNA levels were 1.7 mEq/mL (range 0.6–9.5 mEq/mL). Mean liver/serum ratio (equivalents per gram: equivalents per milliliter) was 103 (range 17.4–286) in the paired specimens in which HCV RNA could be quantified at both sites. HCV RNA was quantitated by bDNA in two of the five patients included in the prospective study, and liver to serum ratios were 253 and 2.8, respectively.

Evaluation of potential sample size limits. Since liver biopsies are often quite small and must be divided for histological testing, we tested the theoretical limits of the size necessary for detection of HCV RNA. Three wedge biopsies from HCV seropositive patients were homogenized and diluted serially (by two-fold dilution) in guanidine-HCl (25 mg/0.5 mL). Diluted samples were processed according to the standard protocol and tested for HCV RNA. Virus was detectable in as little as 1.6 mg of liver tissue from all three specimens.

Reproducibility. In order to evaluate the reproducibility of viral quantitation in liver tissue, multiple aliquots of a homogenized wedge biopsy of a seropositive patient were tested. Results were highly reproducible. Intrarun (four aliquots, one assay) coefficient of variation was 7.7% and interrun variability (one aliquot, four assays) was 11.7%.

Distribution of Viral RNA and Liver Histopathology in Liver Explants

HCV RNA levels in immediate biopsies. Clinical features of the five patients studied prospectively are demonstrated in Table I. Results of HCV RNA quantitated from tissue processed immediately from seven different sites in the livers of five different patients are shown in Figure 2. Geometric mean ± standard deviation from patients 1 through 5 were 480 ± 16, 110 ± 11, 7.7 ± 0.22, 5.3 ± 0.18, and 31 ± 2.2 mEq/g, respectively. The ranges between the highest and lowest measured levels within individual livers differed by a mean of 3.2-fold (0.51 $\log_{10}$) (range 2.0-fold [0.30 $\log_{10}$] to 5.1-fold [0.71 $\log_{10}$]). Within individual livers, 68% of liver quantitations were within 1.5-fold (0.17 $\log_{10}$) of the geometric mean for that liver, and 95% were within 2.2-fold (0.34 $\log_{10}$) of the geometric mean.

HCV RNA levels in delayed biopsies. A mixed-effects analysis of variance showed no significant dif-

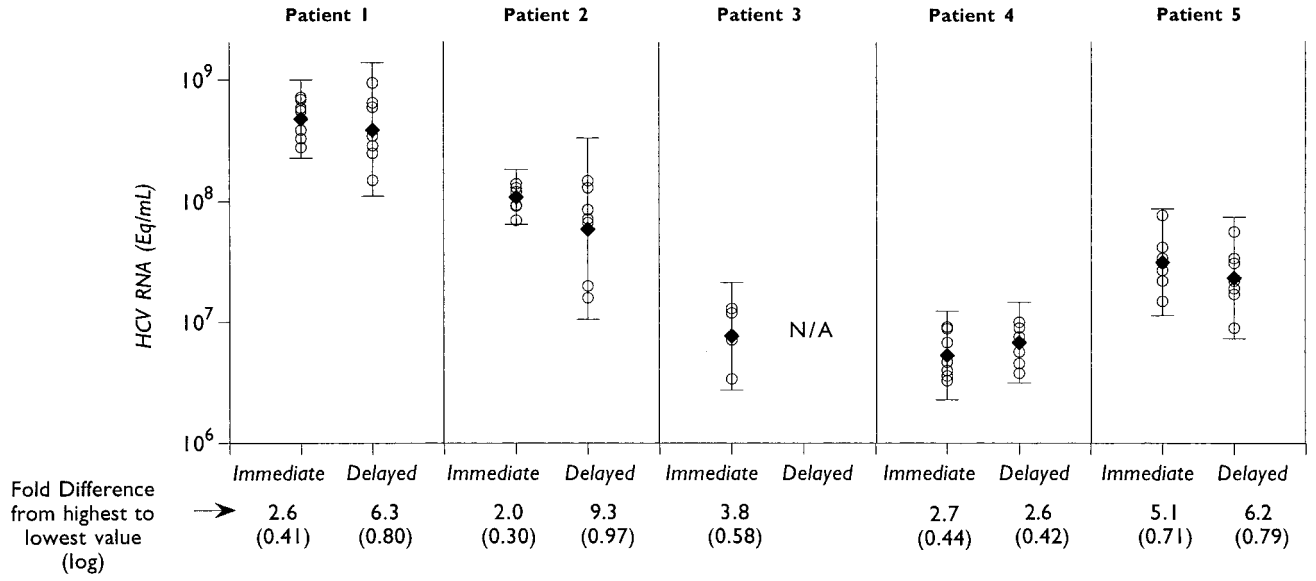

Fig. 2. Quantification of HCV RNA at multiple sites in the liver. HCV RNA levels were measured with the bDNA assay in needle biopsies from seven locations in whole livers from five liver transplant patients. Biopsies from the same locations were taken immediately after removal of the organ (Immediate) and after a 30-minute delay (Delayed). The fold differences and corresponding $\log_{10}$ scale differences in HCV RNA measurements between the highest and lowest HCV RNA quantification values are shown.

ference between viral levels in immediate and delayed specimens (obtained at the same position from seven different locations in the liver at an interval of 30 minutes), nor a consistent association between viral load and specific biopsy location. There was a significant fall in mean level of HCV RNA in only one of four livers tested (Wilcoxon matched pairs test) (Fig. 2). While the standard deviation and range observed in delayed biopsy samples appeared greater in delayed than immediate specimens, this was significant in only one patient ($P = 0.02$; Levene's test for homogeneity of variance).

Histology. Histological score was the same in biopsies taken from seven different locations of the liver of each explant (Table II), and there were no differences in histologic score between specimens taken immediately or after a 30-minute delay (data not shown).

DISCUSSION

The liver is the major site of HCV replication and hence any study which attempts to relate viral load to clinical outcomes should incorporate quantitation of virus in the liver. Yet few studies have included such measurements, a limitation which may contribute to conflicting findings regarding the relationship between viral load and the degree of histological liver damage [Gretch et al., 1994; Nousbaum et al., 1995; Magrin et al., 1994]. Accurate measurement of viral load is important when evaluating pretreatment predictors of response to interferon therapy. Eradication of HCV RNA in the liver may also be an important endpoint of therapy, an endpoint which requires a reliable method for detection of HCV RNA in the liver. Most studies which have examined the predictive value of viral load have measured viral load in serum, largely ignoring viral quantitation in the liver [Shindo et al., 1991; Lau et al., 1993a,b]. The reasons for this include the added risk inherent in obtaining liver specimens compared with serum samples, the lack of standardized methods for processing liver specimens and for efficiently extracting viral RNA, and concerns regarding the validity of determining viral load in a needle biopsy with extrapolation of these results to the liver as a whole. Finally, studies utilizing viral quantitation in the liver are limited by an absence of reliable standards with which to compare results from study to study, and the lack of reliable information on the run-to-run and sample-to-sample variability when specimens are quantitated. In this study, we have addressed many of the issues outlined above.

Liver specimens from patients with end-stage liver disease undergoing orthotopic liver transplantation were used to develop a method for extraction of viral RNA for subsequent quantitation by bDNA. In the extraction of RNA from liver tissue, there are conflicts between maximizing purity and maximizing nucleic acid recovery. Techniques designed to yield purified RNA, because of their complexity, can lead to reduced quantitative recovery. In our initial optimization studies, we were able to obtain efficient recovery of viral RNA with concomitant removal of contaminating cellular DNA. The purity required for bDNA amplification is less than that required for PCR since inhibitors (porphyrins, including hemoglobin) of Taq polymerase, the enzyme used in most PCR reactions, must be removed from the liver for PCR but not for bDNA [Higuchi, 1989]. Thus, we have shown that the guanidine HCl procedure is a simple and efficient means of extracting HCV RNA from liver tissue [Cox, 1968].

Quantitation of extracted RNA by bDNA was both

TABLE II. Liver Histopathology of "Immediate" Biopsies*

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Description[a]	4, 3, 4, 4, 4, 4, 4	5, 4, 4, 4, na, 4, na	4, 4, 4, 4, 4, 4, 4	3, 3, 2, 2, 2, 3, 3	4, 4, 3, 3, 4, 3, 4
Inflammation[b]	3, 2, 3, 3, 3, 3, 3	4, 3, 3, 3, na, 3, na	3, 3, 3, 3, 3, 3, 3	2, 2, 1, 1, 1, 2, 2	3, 3, 2, 2, 3, 2, 3
Fibrosis[c]	4, 4, 4, 4, 4, 4, 4	4, 4, 4, 4, na, 4, na	4, 4, 4, 4, 4, 4, 4	2, 3, 2, 2, 1, 2, 3	4, 4, 3, 4, 4, 3, 4
Lymphoid follicles[d]	–, –, +, +, –, +, –	+, +, +, –, na, +, na	–, +, +, +, +, +, +	–, +, +, +, +, +, +	+, +, +, –, +, +,+
Bile duct lesions[e]	na, +, +, +, +, + +	+, +, +, +, na, +, na	+, +, na, +, +, na, +	–, –, –, –, –, –, –	+, +, +, –, –, –, –

*Biopsy scores for each patient in the following order. Caudate lobe; left lobe sites 1, 2, and 3; right lobe sites 1, 2, ad 3. na, data not available.
[a]Lobular hepatitis = 2; chronic active hepatitis: mild = 3, moderate = 4, severe = 5.
[b]Grade 0 to 4 in increasing severity.
[c]Grade 1 to 4 in increasing severity.
[d]+ (present); – (absent).
[e]+ (present); – (absent).

sensitive and specific, with virus detectable in the liver specimens of 95% of seropositive patients and 0% of seronegative patients. The amount of hepatic viral RNA was similar in specimens obtained by wedge and needle biopsy, and the absolute amounts of viral RNA measured were comparable to those from other studies. Mean HCV RNA levels from wedge biopsies were 87 mEq/g and from needle biopsies were 37 mEq/g, similar to that recently reported in another study of viral quantitation in needle biopsies [140 mEq/g; Idrovo et al., 1996]. Using quantitative RT-PCR methods, the levels of intrahepatic HCV RNA levels which have been reported are 10^8–10^{11} copies/g [Hasui et al., 1994], 10^{10}–10^{11} copies/g [Sugano et al., 1995], and 10^8–10^{11} copies/g [Nakagawa et al., 1994]. The slightly lower levels of liver HCV RNA observed in the present study may be due to the presence of cirrhosis (with implications that viral load falls with advancing liver disease) or may simply be due to differences in RNA standards used for comparison. Some investigators have reported their findings as copies of virus [Sugano et al., 1995; Nakagawa et al., 1994]. Since detection of one unit of viral RNA is not necessarily identical to detection of one intact virus, we have expressed our results in viral equivalents (or million equivalents) throughout. Moreover, since uniform standards have not been used for determining viral levels across studies, results expressed in some studies as "copies" are not necessarily the same as results expressed in "equivalents" in our study.

The ratios of HCV RNA in liver to serum determined in our study was 103 mEq/g:mEq/mL (mean), comparable to ratios found in patients with chronic active hepatitis (CAH; mean 47) and in patients with CAH and CAH/cirrhosis (CAH/C) [mean 36; Idrovo et al., 1996; Sugano et al., 1995]. Other investigators have reported higher liver to serum ratios [100 and 1,000; Hasui et al., 1994; Nakagawa et al., 1994; Yatsuhashi et al., 1995]. Assuming that it is appropriate to compare 1 mL of serum with 1 g of liver tissue, the relatively high HCV RNA liver/serum ratios reported in all of these studies suggest that HCV RNA which is present extracellularly is a small proportion of the total amount of HCV RNA. Given the expense and risk entailed with liver biopsy, a standard "conversion factor" which would enable calculation of hepatic viral content from results in serum would be useful. While correlations between levels of liver and serum RNA have been demonstrated by several investigators [Idrovo et al., 1996; Coelho-Little et al., 1995; Nakagawa et al., 1994; Yatsuhashi et al., 1995], no accurate numerical constant or conversion factor has been defined. In fact, as detailed above, the ratios of liver vs. serum viral load vary widely over a three-log range.

A typical needle biopsy specimen, which weighs between 10 and 50 mg, provides sufficient liver tissue both to measure HCV RNA levels and to evaluate histopathology. In the present study, HCV RNA could be detected reliably by bDNA assay in as little as 2 mg of liver tissue. The levels of HCV RNA in the biopsy specimens from seropositive patients were such that a minimum of 2.5 mg of liver tissue would have been sufficient to detect HCV RNA with the bDNA assay in all but one case.

It has been estimated that a typical needle biopsy contains 1/63,000 of the volume of the liver [Feldmann, 1995]. Thus, there is potential that determination of hepatic viral levels from a needle biopsy may not be representative of levels of virus in the rest of the liver. Analysis of HCV RNA levels in needle biopsies from different locations of liver explants revealed a homogeneous distribution in the liver. Of all immediate needle biopsies, 95% were within 2.2-fold (0.34 log difference) and 67% were within 1.5-fold (0.17 log difference) of the mean. This degree of variability in HCV RNA levels between specimens is similar to that reported in a study comparing right and left lobes of the liver in noncirrhotic patients [Idrovo et al., 1996]. In spite of the fact that all of the patients in the present study had cirrhosis, which is believed to increase sampling variability for liver histology [Feldmann, 1995], there was good concordance between HCV RNA levels as well as histopathology measured at multiple sites. There was also no statistically significant difference between hepatic HCV RNA levels taken immediately after removal, and those obtained after a 30-minute delay.

While immediate freezing of liver tissue may not always be necessary, it would seem prudent to minimize the duration of time that biopsy specimens are maintained at room temperature.

In our study, frozen wet weight of liver biopsies was used as a reference point, or denominator, by which to standardize measurements of HCV RNA in the liver. HCV RNA levels were expressed as HCV RNA mEq/g. We chose frozen wet weight of liver biopsies as the denominator because it is simple, and because it allows a rational comparison between serum HCV RNA levels (per milliliter) and liver HCV RNA levels (per gram). Other studies have used both frozen wet weight [Idrovo et al., 1996; Coelho-Little et al., 1995; Yatsuhashi et al., 1995; Sugano et al., 1995] and total RNA [McGuinness et al., 1996; Sakamoto et al., 1995] and total RNA per gram of tissue [Hasui et al., 1994; Nakagawa et al., 1994]. Because of concerns with the variability in recovery of RNA from tissue samples, other investigators have used levels of β-actin mRNA as an internal standard of reference for each sample [Grassi et al., 1995]. There are important limitations in using either frozen wet weight or total RNA measurements as a reference point. Total RNA determination by measurement of OD_{260} may be inaccurate because highly purified preparations are required to avoid protein and DNA contamination [Sakamoto et al., 1994]. Since the extraction methods used to achieve high-purity RNA are complex, there can be substantial loss and variability in yield of RNA. There are also potential problems with the use of frozen wet weight of liver tissue as a denominator since the relative proportion of weight contributed by nonparenchymal cells and hepatocytes may be different in cirrhotic and noncirrhotic liver tissue. As an alternative approach to be explored in future studies, total cellular DNA levels as well as wet weight may be used as the reference point [Meyer et al., 1991].

Currently, there are insufficient data on the superiority of measurement of viral load in liver tissue over measurement in serum, so liver biopsy for this indication alone cannot be advocated. Further clinical research will be required to evaluate the predictive value of hepatic viral load.

In summary, we have developed a simple and efficient method to extract HCV RNA from liver tissue which can then be reliably detected and quantified by the bDNA assay. The amount of liver tissue present in needle biopsies is sufficient to quantify HCV RNA, even when the specimen is divided for histological analysis. HCV RNA is homogeneously distributed in the liver, supporting the hypothesis that viral load measured in a single biopsy is representative of the liver as a whole. While there was no significant fall in level of HCV RNA in samples which were processed after a delay, it seems prudent to freeze tissue as rapidly as possible. Further study of HCV RNA levels in liver tissue may contribute to our understanding of the pathogenesis of HCV-associated liver disease and yield valuable insight into the clinical management of patients with chronic infection.

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